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(54) **apoAI-CIII-AIV, apoAII apoB, apoCI, and LDL receptor polymorphisms for genetic fingerprinting and predictive of atherosclerosis.**

(57) The invention offers a method of genetic analysis which is potentially an early detection method for atherosclerosis. The method comprises detecting the presence or absence of polymorphic states correlated with this disease which are proximal to the apolipoprotein AI-CIII-AIV gene complex, or to the apoAII, apoB, apoCI or, LDL-R genes. Individuals with particular polymorphic sites are likely to experience severe atherosclerotic symptomologies.

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apoAI-CIII-AIV, apoAII apoB, apoCI, AND LDL RECEPTOR POLYMORPHISMS FOR GENETIC FINGERPRINTING AND PREDICTIVE OF ATHEROSCLEROSIS

Technical Field

The invention relates to the use of genetic polymorphisms to determine disease states. More particularly, the invention concerns the use of polymorphisms of the apolipoprotein AI-CIII-AIV gene region, and of the apoB, apoCI, apoAII and LDL receptor genes, to diagnose susceptibilities to atherosclerosis.

Background Art

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The degree of morbidity and mortality associated with atherosclerosis in developed countries is higher than that associated with any other particular disorder, even cancer. The disorder manifests itself in the form of cholesterol deposition in arterial cell walls. The deposition is slow and irreversible and starts at an early age. Clinical symptoms may take years to manifest themselves and are extremely serious; they include coronary heart disease and stroke. Generally, the disease process will have begun long before these clinical manifestations appear.

Because environmental as well as hereditary factors influence the course of the cholesterol deposition and offer means for at least a mitigation of the process, it is desirable to have available a diagnostic technique which provides an early warning of the onset of the deposition. The present technique depends on measuring cholesterol or triglyceride levels in serum, and while these levels are, indeed, capable of accurate measurement, they do not offer the desirable high correlation to true susceptibility. More reliable predictive methods, which rely on detection of atheromatous lesions, use highly invasive procedures, which are sufficiently painful and expensive that they cannot be employed on a screening basis, or even applied to specific groups selected on the basis of family histories. These techniques also offer too little, too late; by the time the atheromatous lesions have appeared, the most effective time for treatment has been passed.

The importance of early detection is made more poignantly evident by the fact that an effective, but inconvenient and unattractive long term treatment is available—i.e., lowering serum cholesterol through consistently controlled diet or use of cholesterol-lowering drugs. Resistance to this approach will be encountered unless it is clear that the "deprivation" is warranted. The problem is not what the treatment should be, but to whom the treatment should be applied.

A technique that inherently offers the advantages of early detection, if its results can be effectively correlated with the disorder to be assessed, is genetic analysis. Since the genomic characteristics of an individual are basically determined, it is assumed, at conception, genetic aberrations which are indicia of later metabolic disorders are an ideal early diagnosis tool. Genetic testing can be routinely conducted using present methodology, as early as the seventh week of fetal life. Over the last ten years, the availability of restriction enzymes and DNA probing techniques has made possible the use of "restriction fragment length polymorphisms" (RFLPs) in such diagnosis. Using the by now well established Southern blot hybridization technique (Southern, E., *J Mol Biol* (1975) 98:503-517), it has been possible successfully to diagnose sickle cell anemia (Kan, Y.W., et al, *Proc Natl Acad Sci (USA)* (1978) 75:5631);  $\beta$ -thalassemia (Antonarakis, S.E., et al, *Proc Natl Acad Sci (USA)* (1983) 79:137); type II diabetes (Rotwein, P., et al, *Science* (1981) 213:1117); familial growth hormone deficiency (Phillips, J.A., III *Banbury Report* 14, Cold Spring Harbor Laboratory (1983) pp 305-315); phenylketonuria (Woo, S.L.C., et al, *Nature* (1983) 306:151); Huntington's disease (Gusella, J.F., et al, *Nature* (1983) 306:234); and hemophilia B (Gianelli, et al, *Lancet* (1984) i:239, Grunenbaum, et al, *J Clin Invest* (1984) 73:1491).

All the foregoing successes are grounded in the identification of a particular polymorphism or polymorphisms which correlates with the disease or disorder in question. It should be noted, however, that the technique is also useful from a more general standpoint in genetically fingerprinting individuals, e.g. in paternity testing or the like.

It has been calculated that the number of polymorphisms expected in the human genome should be of the order of  $10^7$ , based on an assumed probability of 0.05 for a given nucleotide to be polymorphic; this number being inferred from studies of the human growth hormone,  $\alpha$ -antitrypsin and  $\beta$ -like globin gene cluster loci (Jeffreys, A. J., *Cell* (1979) 18:1-10; Oster, H., et al, *Am J Hum Gen* (1984) 36(suppl) 150S). In detecting or predicting disease, the challenge is to determine which of these over ten million polymor-

phisms is associated with a particular disorder, and to devise a procedure to detect it. The present invention has met this challenge with regard to atherosclerosis, and has also identified a pattern of polymorphisms associated with the same locations in the genome useful in characterizing particular individuals and patterns of inheritance.

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### Disclosure of the Invention

The invention provides identification of polymorphisms which are predictive of the subsequent develop-  
 10 ment of atherosclerosis or which are indicative of protection against development of this disease, and include polymorphisms in the regions of the genome which encode apolipoproteins AI, CIII and AIV (the apoAI-CIII-AIV gene complex), (and which encode apolipoproteins apoAII, B, and CI (the apoAII, apoB and apoCI genes). The invention also includes identification of polymorphisms in the region of the genome encoding the receptor for low density lipoprotein (LDL-R gene). The apoAI-CIII gene region of the apoAI-CII-  
 15 AIV complex is described in applicant's copending applications U.S. Serial Nos. 782,666, filed 30 September 1985, 900,593, filed 26 August 1986, 924,911, filed 29 October 1986, and 044,449, filed 30 April 1987. The disclosure of each of these co-pending applications is hereby incorporated by reference.

The polymorphisms of the present invention, taken together or in subsets, can provide an identity profile of a particular individual, thus providing a means to follow family inheritance patterns and to assess  
 20 relationships between individuals. In addition, since these polymorphisms are located in the genomic sequences which regulate lipid metabolism, it is probable that their pattern of presence or absence will show some additional specific, as yet unknown, correlation to symptomology.

In one aspect, then, the invention is directed to a method of genetically identifying an individual, in particular with respect to predicting the likelihood of development of atherosclerosis in said individual, which  
 25 method comprises:

- 1 detecting the presence or absence of a polymorphic site in the third intron of the apolipoprotein AI (apoAI) gene detectable in a BanI digest (the "BanI-apoAI" polymorphism); and/or
- 2 detecting the presence or absence of a polymorphic site in the first intron of the apoAI gene detectable in an MspI digest (the "MspI/0.73-apoAI" polymorphism); and/or
- 30 3 detecting the presence or absence of a polymorphic site in the third intron of the apoAI gene detectable in a BanII digest (the "BanII-apoAI" polymorphism); and/or
- 4 detecting the presence or absence of a polymorphic site in the third intron of the apoAI gene detectable in a BsmI digest (the "BsmI-apoAI" polymorphism); and/or
- 5 detecting the presence or absence of a polymorphic site 1.0 kb 3' of the apoAI gene detectable in a  
 35 BstXI digest (the "BstXI-apoAI" polymorphism), and/or
- 6 detecting the presence or absence of a polymorphic site at the apoAII gene locus detectable in a BstEII digest (the "BstEII-apoAII" polymorphism), and/or
- 7 detecting the presence or absence of a polymorphic site at the apoB gene locus detectable in an HaeIII digest (the "HaeIII-ApoB" polymorphism), and/or
- 40 8 detecting the presence or absence of a polymorphic site at the apoB gene locus detectable in an MvaI digest (the "MvaI-ApoB" polymorphism), and/or
- 9 detecting the presence or absence of a polymorphic site at the apoB gene locus detectable in an NsiI digest (the "NsiI-ApoB" polymorphism), and/or
- 10 detecting the presence or absence of a polymorphic site at the apoB gene locus detectable in an HpaI  
 45 digest (the "HpaI-ApoB" polymorphism), and/or
- 11 detecting the presence or absence of a polymorphic site at the apoCI gene locus detectable in a BglI digest (the "BglI-apoCI" polymorphism), and/or
- 12 detecting the presence or absence of a polymorphic site at the apoCI gene locus detectable in a DraI digest (the "DraI-apoCI" polymorphism), and/or
- 50 13 detecting the presence or absence of a polymorphic site at the apoCI gene locus detectable in a TaqI digest (the "TaqI-apoCI" polymorphism), and/or
- 14 detecting the presence or absence of a polymorphic site at the LDL-R gene locus detectable in a Cfr13I digest (the "Cfr13I-LDL" polymorphism), and/or
- 15 detecting the presence or absence of a polymorphic site at the LDL-R gene locus detectable in a BstEII  
 55 digest (the "BstEII-LDL" polymorphism).

Stated alternatively, the invention is directed to a method for genetic fingerprinting, in particular, detecting susceptibility to atherosclerosis which comprises:

- 1 detecting a 1.46 kb vs. both a 0.82 kb and a 0.64 kb BanI digestion fragment hybridizing to the apoAI

probe, or its substantial equivalent; and/or

detecting a 0.73 kb vs. 0.65 kb MspI digestion fragment hybridizing to the apoA1<sup>1</sup> probe, or its substantial equivalent; and/or

5 detecting a 0.452 kb vs. 0.274 kb BanII digestion fragment hybridizing to the apoA1<sup>2</sup> probe, or its substantial equivalent; and/or

detecting a 6.9 kb vs. 5.6 kb BsmI digestion fragment hybridizing to the apoA1<sup>1</sup> probe, or its substantial equivalent, or the presence or absence of a 9.3 kb fragment hybridizing to apoCIII probe or its substantial equivalent; and/or

10 detecting a 4.5 kb vs. 1.8 kb BstXI digestion fragment hybridizing to the apoCIII probe, or its substantial equivalent, or a 4.5 kb vs. 2.7 kb BstXI digestion fragment hybridizing to an apoA1<sup>1</sup> probe or its substantial equivalent; and/or

detecting a 4.8 kb vs. 6.5 kb BstEII digestion fragment hybridizing to the apoAII probe, or its substantial equivalent; and/or

15 detecting an HaeIII digestion fragment of variable size between 0.9 kb and 1.1 kb hybridizing to the 3'-apoB probe or its substantial equivalent; and/or

detecting a 0.96 kb vs. 0.76 kb MvaI digestion fragment hybridizing to the 3'-apoB probe or its substantial equivalent; and/or

detecting a 3.8 kb vs. 6.2 kb NsiI digestion fragment hybridizing to the 5'-apoB probe, or its substantial equivalent; and/or

20 detecting the presence or absence of a 15.1 kb HpaI digestion fragment hybridizing to the 5' apoB probe or its substantial equivalent; and/or

detecting an 8.7 kb vs. 6.2 kb BglI digestion fragment hybridizing to the apoCI probe, or its substantial equivalent; and/or

25 detecting a 10.2 kb vs. both a 7.6 kb and a 2.6 kb DraI digestion fragment hybridizing to the apoCI probe, or its substantial equivalent; and/or

detecting the presence or absence of a 5.9 kb TaqI digestion fragment hybridizing to an apoCI probe or its substantial equivalent; and/or

detecting a 3.0 kb vs. 4.0 kb Cfr13I digestion fragment hybridizing to the LDL-R probe or its substantial equivalent; and/or

30 detecting both a 22 and 11 kb vs. a 33 kb BstEII digestion fragment hybridizing to the LDL-R probe, or its substantial equivalent.

The invention is also directed to kits suitable for performing the methods of the invention.

### 35 Brief Description of the Drawings

Figure 1 shows a map of twelve polymorphisms found in the apoA1-CIII-AIV gene complex.

Figure 2 gives the DNA sequence for the apoB probe used in detecting some of the polymorphisms disclosed herein.

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### Modes of Carrying Out the Invention

45 In the description below, distances of polymorphisms from reference points and lengths of deletions are often given in bp or kb. Where the sequence is known, such measures can be quite precise, but when assessed by measuring fragment sizes on gels or by other experimental means, these measures contain a margin of uncertainty, as is well understood in the art. In general, for measures of >4 kb, the margin of uncertainty is  $\pm \sim 0.3$  kb; for smaller lengths, the error is  $\pm \sim 10\%$ . Thus, the "300 bp" deletion may be slightly larger or smaller, and the 4 kb spacing from the apoA1 gene is only approximate.

50 The nomenclature used to identify the polymorphisms disclosed herein is as follows. Generally, each polymorphism is identified with the restriction enzyme that cleaves a particular segment of the genome to give either a "normal" (more common allele) or a "polymorphic" (less common allele) fragment.

Where digestion with a given restriction enzyme yields more than one polymorphism, each polymorphism is then also identified by the length of the polymorphic fragment--e.g., "MspI/0.73 apoA1".

55 Related polymorphisms not discussed at length herein are disclosed in applicant's copending applications previously incorporated by reference.

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# EUROPEAN SEARCH REPORT

Application Number

EP 87 30 9482

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D, X	WO-A-8 606 102 (BIOTECHNOLOGY RESEARCH PARTNERS Ltd.) * Whole document *	1-4	C 12 Q 1/68 C 12 N 15/00 C 12 P 19/34
D, P X	WO-A-8 702 059 (BIOTECHNOLOGY RESEARCH PARTNERS Ltd.) * Whole document *	1-4	
X	CHEMICAL ABSTRACTS, vol. 105, September 1986, page 175, abstract no. 92353e, Columbus, Ohio, US; R.T. COLEMAN et al.: "Polymorphisms in the apolipoprotein AI-CIII gene complex", & MOL. BIOL. MED. 1986, 3(3), 213-28 * Abstract *	1-4	
A	NUCLEIC ACIDS RESEARCH, vol. 14, no. 19, October 1986, page 7820, IRL Press Ltd, Oxford, GB; H. FUNKE et al.: "Pst RFLP close to the LDL receptor gene"		
Y	NUCLEIC ACIDS RESEARCH, vol. 14, no. 10, May 1986, page 4373, IRL Press Ltd, Oxford, GB; P.M. FROSSARD et al.: "Pvu II RFLP in the 5' of the human apolipoprotein B gene" * Whole document *	1-4	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)  C 12 Q C 12 N
Y	DNA, vol. 3, no. 4, 1984, pages 309-317, Mary Ann Liebert, Inc. Publishers; J.J. SEILHAMER et al.: "Isolation and DNA sequence of full-length cDNA and of the entire gene for human apolipoprotein AI-discovery of a new genetic polymorphism in the apo AI gene" * Whole document *	1-4	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 15-02-1988	Examiner OSBORNE H.H.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document			

EP 87 30 9482 (1988)

### A. Techniques for Detection of Polymorphisms

Application of the method of the invention to predict potential atherosclerotic individuals or to obtain a genetic "fingerprint" based on some or all of the polymorphisms associated with the general genomic apoA1-CIII-AIV complex and apoB and CI genes, employs standard techniques of DNA extraction, purification, restriction enzyme digestion, and size separation. Techniques for hybridization using probes and successful detection of hybridized substrate arranged according to molecular weight are also well known to those in the art. The general approach to finding and detecting the significant polymorphisms is the following:

DNA is extracted from the somatic cells of the individual to be tested, for example from leukocytes, placental cells, cultured fibroblasts, or, in the case of fetal individuals, from cells of the amniotic fluid. The high molecular weight DNA fraction is separated, and subjected to treatment with a particular, selected restriction enzyme, such as, for example, EcoRI, BamHI, MstI, XmnI, and the like. After digestion of the high molecular weight DNA, the digest is applied to a polyacrylamide or agarose gel and subjected to electrophoresis to obtain separation of the DNA fragments resulting from restriction enzyme digestion into positions on the gel determined by the size (length) of the fragment. The contents of the gel are then replicated by transferring to a nitrocellulose filter or other suitable matrix for use as a probe hybridization support. The DNA fragments, either before or after transfer to the nitrocellulose filter replica, are treated with a denaturant such as sodium hydroxide/salt. The denatured, single-stranded DNA, replicated electrophoresis patterns are probed with labeled (usually by  $^{32}\text{P}$ ) single-stranded DNA fragments. Other labels besides radioactivity, such as fluorescent molecules may also be used. The complex formed with probe may also be detected by methods designed to detect the complex per se, rather than to label either component, such as, for example, binding to labeled antibodies specific for double-stranded nucleic acids.

When probes are labeled by nick translation using, for example,  $\alpha[^{32}\text{P}]$  dCTP and  $\alpha[^{32}\text{P}]$  dGTP, which results in fragmentation of the probe, cDNA probes which are complementary only to the exon regions of the gene and which span over intron regions are readily workable in the method of the invention.

Depending on the probe selected, fragments will be detected which derive from a particular region on the genome. For example, in the method of the invention, a cDNA sequence from the apolipoprotein AI (apoAI), apolipoprotein AII (apoAII), apolipoprotein B (apoB), apolipoprotein CI (apoCI), or LDL receptor (LDL-R) gene sequences is used as a probe. Therefore, the only fragments which will appear on the hybridized filters are those which contain sequences complementary to this probe--i.e., only those which have not been severed either in the genome itself or by the restriction enzyme cleavage from the complementary apoAI, apoAII, apoB, apoCI or LDL receptor fragment. Stated in another way, by using a particular probe, alterations in the genome which are proximal to sequences corresponding to that probe are detected.

The specific procedures used in the general process described in the preceding paragraphs are understood in the art. Procedures for DNA extraction from somatic cells may, for example, be found in Kan, Y.W., et al, Proc Natl Acad Sci (USA) (1978) 75:5631-5635; Taylor, J.M., et al, Nature (1984) 251:392-393, and Kan, Y.W., et al, N Eng J Med (1977) 297:1080-1084. Further improvements which permit rapid extraction of the DNA are also disclosed by Law, D. G., et al, Gene (1984) 28:153-158. Techniques for size separation of the restriction enzyme treated DNA fragments are also described in the foregoing references. Restriction enzyme digestions are generally standard in the art and are carried out under buffer, ionic strength, and temperature conditions which are specified by the manufacturer of the particular restriction enzyme.

Transfer to nitrocellulose or other support and probing by prehybridization with nonspecific DNA followed by hybridization with labeled probe are also standard procedures disclosed, for example, in the foregoing references and by Southern, E., (supra). The section of the genome which is fingerprinted or otherwise subject to study using the results is, of course, dependent on the nature of the probe. The probes useful in the present invention are selected from the apoA1-CIII-AIV gene complex, and from the apoAII, apoB, apoCI, and LDL receptor genes.

### B. Nature of the Probes Useful in the Invention

The fragment pattern obtained is diagnostic for a particular polymorphism if the probe selected is complementary to a DNA sequence sufficiently proximal to the polymorphism on the genome that it is not severed from the polymorphism by the restriction cleavage, and has a low probability of being segregated from the polymorphism by crossing over. Acceptable distance limits between the region of probe

complementarity and the polymorphism are therefore arbitrary. Generally, probes which hybridize to DNA sequences within 10 kb upstream or downstream of the polymorphism give acceptable results. Occasionally, the pattern of restriction enzyme cleavage may place a distal probe hybridization site on a fragment irrelevant to the polymorphism. The closer the probe to the polymorphism, the greater the range of usable restriction enzymes. Accordingly, as used herein, a probe which is a "substantial equivalent" to a specified probe is one which hybridizes to the same fragment length in digests of DNA from individuals with a particular polymorphism when the same restriction enzyme is used. For example, in the detection of the XmnI/8.2 kb polymorphism of the apoA1 gene, used as a model for atherosclerosis-related polymorphisms, two probes described below, an apoA1 probe and a p5'A1 probe, are equivalent when XmnI is used as a restriction enzyme. For detection of this polymorphism using RsaI digestion, they are not. (Of course, the designated probe can be modified in a trivial manner by being made longer or shorter or by selecting a slightly displaced sequence.)

Since atherosclerosis is associated with a defect in cholesterol metabolism, the apoA1-CIII-AIV gene complex which is associated with regulation of blood plasma cholesterol is a region of appropriate probe complementarity. Regulation of blood plasma cholesterol is related to high density lipoprotein (HDL), which is known to be a carrier of cholesterol in the bloodstream, and thus believed to play a significant role in regulating its level--apoA1 is the major structural protein of HDL. It is a 243 amino acid polypeptide having a molecular weight of 28,000. In addition to being a structural member of the HDL complex, apoA1 is also an activator of lecithin-cholesterol acyltransferase, which enzyme is responsible for transforming the lamellar surface structures of chylomicrons into pseudomicellar spherical HDL by *de novo* synthesis of cholesterol esters and lysolecithin. The apoA1 protein may also be the HDL component recognized by the HDL receptors.

Accordingly, the apoA1 gene region is a portion of the genome where modification might occur in individuals with a prognosis for atherosclerosis or related disorders. This gene lies close to a related gene encoding apolipoprotein CIII (apoCIII) which is 2.5 kb from the apoA1 gene on the long arm of chromosome 11 (Cheung, p., et al, Proc Natl Acad Sci (USA) (1984) 81:508-511; Law, S. W., et al, Biochem Biophys Res Comm (1984) 118:934-942). The apoAIV gene is located 7.8 kb 3' to the apoCIII gene and 12.5 kb 3' to the apoA1 gene (Karathanasis, S.K., Proc. Natl. Acad. Sci. USA (1985) 82:6374-6378). This region of the genome is herein designated the "apoA1-CIII-AIV complex". Genomic and cDNA clones for apoA1, apoCIII and apoAIV have been isolated by, e.g., Karathanasis, S. K., et al, Proc Natl Acad Sci (USA) (1983) 80:6147-6151; Shoulders, S. C., et al, Nucleic Acids Res (1983) 11:2827-2837 and by others. Various apoA1 and apoCIII probes have been described (see, e.g., Karathanasis, S.K., et al (supra); Shoulders, S.C., et al (supra)) and have been used previously as hybridization probes to detect polymorphisms.

Karathanasis, S. K., et al, (Nature (1983) 301:718-720; *ibid.* (1983) 305:823-825) showed a 6.5 kb DNA insertion in the coding region of apoA1 in one family suffering from HDL deficiency. Rees, A., et al, Lancet - (1983) i:444-446 showed an SstI polymorphism in the 3' flanking region of the apoCIII gene which was present in some hypertriglyceridemic patients. Seilhamer, J.J., et al, DNA (1984) 3:309-317 have disclosed an MspI polymorphism in the third intron of the apoA1 gene.

Apolipoprotein AII is a 77 amino acid polypeptide and is a component of HDL. Although the functional role of apoAII remains unclear, it might regulate the activities of lecithin-cholesterol acyltransferase (Scanu, A.M., et al, Ann NY Acad Sci (198 ) 348:160-173) and hepatic lipase (Shinomiya, M., et al, Biochim Biophys Acta (198 ) 713:292-299; Scott, Y., et al, Lancet (1985) 1:770-773); have described the presence of an MspI polymorphic site 3' to the apoAII gene, which correlates with increased serum apoAII levels.

Apolipoprotein B is the major protein component of very low density lipoproteins (VLDL) and of chylomicrons. It is the sole protein in low density lipoproteins (LDL), and is essential for the assembly and secretion of chylomicrons and VLDL. It also functions as the ligand for removal of LDL from circulation by receptor-mediated uptake into a variety of cells. (Lusis, A.J., et al, Proc Natl Acad Sci (USA) (1985) 82:4597-4601.) Four major plasma species of apoB have been described (Kane, J.p., et al, Proc Natl Acad Sci (USA) (1980) 77: 2465-2469). However, two of these appear to arise from one of the others by virtue of the protease activities found in plasma. (Cardin, A.D., et al, J Biol Chem (1984) 259: 8522-8528; Yamamoto, M., et al, J Biol Chem (1985) 260: 8509-8513). One of the primary forms, apoB-48, is synthesized by the intestine and is a component of chylomicrons; the other primary form, which is apparently attacked by the plasma protease, apoB-100, is the protein ligand on LDL that binds to the LDL receptor and results in uptake and catabolism of LDL by the liver (Deeb, S.S., et al, Proc Natl Acad Sci (USA) (1985) 82: 4983-4986). In any event, the apolipoproteins encoded by the apoB gene are integral to cholesterol and fat metabolism. Indeed, it has been shown by others that elevated plasma levels of apoB-100 have been found in individuals with premature coronary artery disease (Brunzel, J.D., et al, Atherosclerosis (1984) 4: 79-83). and individuals with familial hyperlipidemia and hypercholesterolemia also seem to have elevated levels of

this protein (Brunzel, J.D., et al, *ibid.*; Brunzel, J.D., et al, *J Lipid Res* (1983) 24: 147-155). At least partly because of this interest, cDNA clones for apoB or portions thereof have been prepared (Deeb, S.S., et al, and Lusis, A.J., et al, both *supra*; Protter, A.A., et al, *Proc Natl Acad Sci*, in press). Thus, a portion of the apoB gene is useful as a probe to detect polymorphisms in this area, as will be described.

The apoC1 gene encodes the major protein component of human very low density lipoprotein, and a minor component of high density lipoprotein. These factors are also intimately involved in lipid metabolism. In addition, apoC1 binds to phospholipid and can activate lecithin cholesterol acyltransferase (L-CAT) *in vitro* in a manner similar to that of apoA1. Thus, apoC1, like the apoA1-CIII-AIV complex, is intimately involved in cholesterol metabolism (Davison P.J., et al, *Biochem Biophys Res Comm* (1986) 136: 876-884). In addition, it is shown in the Davison paper that there appear to be two apoC1 gene loci which are located on the same chromosome. These loci apparently have sufficient homology that a standard apoC1 probe would bind to either.

Thus, also useful as a probe is the apoC1 region, which encodes apolipoprotein C1. A suitable probe for detection of polymorphisms in this region is the "apoC1" probe, a 300 bp apoC1 cDNA isolated from a fetal liver library, and having a sequence exactly identical to that contained in the sequence published by Knott, T.J., et al, *Nucleic Acids Res* (1984) 12:3909-3915, shown in Figure 3 thereof.

As discussed above, LDL receptor mediates uptake of LDL from the circulation. Elucidation of LDL receptor function may facilitate understanding the mechanism of diseases associated with defective receptor function, such as atherosclerosis. Human low density lipoprotein receptor (LDL-R) DNA has been isolated (Yamamoto et al, *Cell* (1984) 39:27-38), and a probe may be obtained as described in detail below.

The apoA1<sup>1</sup>, apoA1<sup>2</sup>, 5'A1, apoCIII, apoAII, 5'-apoB, 3'-apoB, apoC1, and LDL-R probes which illustrate those useful in the invention are described in detail below. Any probes substantially equivalent to these for a specific restriction enzyme, as defined above, may be used.

The relationship of the probe/enzyme combination to results can be illustrated using a known atherosclerosis predictive polymorphism, which consists of an approximately 300 bp deletion about 4 kb 5' of the apoA1 gene. The deletion is detectable, for example, by digesting the extracted cellular DNA with XmnI, subjecting the fragments to electrophoresis gel separation, and probing with either apoA1 or p5'A1 probes. All persons showing an 8.2 kb fragment are at high (perhaps 100%, risk of developing atherosclerosis, if, indeed, the symptoms have not already appeared. Because of the pattern of XmnI restriction sites in this region, the apoA1 and p5'A1 probes are substantial equivalents in this test. Based on this pattern, one would expect that substantially equivalent probes would be found within 10 kb of the polymorphism, i.e., within about 14 kb upstream or 4 kb downstream of the apoA1 gene with respect to digestion with XmnI. These two probes are, however, not equivalent with respect to a series of additional restriction enzymes which are capable of detecting this polymorphism but which cut either closer to the polymorphism or on the side of it opposite the apoA1 gene. For such restriction enzymes, probes equivalent to p5'A1 must be located between the restriction site and the polymorphism.

### C. Atherosclerosis-Related Polymorphisms

#### The XmnI/8.2 kb Polymorphism

One abnormal allele associated with atherosclerosis, i.e., that containing the abovementioned 300 bp deletion, results in an 8.2 kb XmnI digest fragment, detectable with the first apoA1 probe or with the p5'A1 probe, rather than the 8.5 kb fragment which is obtained "normally". The definition of "normal" in the foregoing sense has to do with the nature of the disease being diagnosed and describes a genomic makeup which does not dispose the subject to the disorder. The majority of the total population will be "normal".

To correlate the genetic test results with atherosclerosis, 125 individuals were tested using XmnI and apoA1 or p5'A1 probe. DNA from ten of these individuals contained the 300 bp deletion, i.e., yielded the 8.2 kb fragment. All of these ten had previously suffered a myocardial infarction; furthermore the presence of the deletion correlated with low levels of HDL cholesterol, the major feature of familial combined hyperlipidemia, a disease associated with early development of severe atherosclerosis and its subsequent clinical symptoms. Twenty-five of the individuals tested were normal control healthy individuals; the DNA from none of these persons contained the deletion. The remaining ninety tested individuals (also not showing the 8.2 kb fragment) were known to have atheromatous plaques as detected by angiography, but not all had suffered heart attacks. Thus, in this sample the deletion is present only in individuals at serious risk from

atherosclerosis. It is not present in all such individuals. Therefore, a positive test using this polymorphism is diagnostic and predictive and preventive methods of therapy would be clearly appropriate. (Of course, a negative test may not mean that the individual is at no risk; it may merely indicate that other genetic defects or other factors predispose that individual to the atherosclerosis problem.)

#### D. Additional Polymorphisms

The polymorphisms of the invention associated with the apoAII-CIII-AIV complex or with the apoAII, apoB, apoCII or LDL-R gene loci may also be analyzed using a group of individuals similar to that discussed in 1C above. This group is divided into atherosclerosis "patients" --i.e., those persons shown to have atheromatous plaques as detected by angiography, whether or not they had heart attacks; and "controls", which consist of those with negative results in this test. Use of this division permits statistical analysis of data to assign increased or decreased risk of atherosclerosis, depending on the presence or absence of particular polymorphisms.

The polymorphisms of the invention are set forth along with the XmnI/8.2 kb polymorphism in Table 2 of the examples below. Some of these polymorphisms have been characterized in terms of their location; however, all can be defined operationally as detectable by digestion by a particular restriction enzyme and probing with a specific probe or its substantial equivalent. The pattern of the presence or absence of these polymorphisms is capable of fingerprinting an individual genetically so that hereditary patterns between parents and progeny can be traced. Also, because these polymorphisms are associated with regions of the genome important in lipid metabolism and transport, the presence of one or, more probably, several of these polymorphisms is likely to be associated with a propensity to disorders of lipid metabolism and related symptomologies. At the present time, limited precise correlations have been made between the presence of particular polymorphisms in this group and particular symptomologies. However, ascertaining the presence or absence of these genetic modifications is useful for the general purpose of identifying individuals with presumptively high risk of lipid disorder symptomologies and for providing a genetic characterization of individuals and family patterns.

#### E. Kits

The reagents suitable for applying the method of the invention to detect the appropriate polymorphisms may be packaged into convenient kits providing the necessary materials, packaged into suitable containers, and, optionally, suitable containers or supports useful in performing the assay. The essential components of the assay include the restriction enzyme associated with the polymorphism, and a suitable probe. Additionally, packages containing concentrated-forms of reagents used for hybridization, prehybridization, DNA extraction, etc. may be included if desired. In particular, however, labeled probe, or reagents suitable to form conveniently labeled probe, are useful in facilitating the conduct of the method of the invention.

#### F. Examples

The following examples are specific with respect to the probes exemplified and with respect to the precise conditions of DNA extraction, probe hybridization, etc. It is understood that these factors are illustrative but not limiting. The essential features of the invention as it relates to detection of a particular polymorphism are selection of enzyme and probe. For example, in detecting the standard XmnI/8.2 deletion, one may use XmnI digestion of the genomic DNA and probe with a sequence complementary to the genomic sequence (in the nonrepeating regions) proximal (i.e., within ~ in this case < 8.2 kb) to the site of the polymorphism. Alternatively, other restriction enzymes may be used in conjunction with a probe which hybridizes in particularly close proximity to the polymorphism.

The various polymorphisms of the invention may be detected using other specific restriction enzymes than those illustrated below. A variety of substantially equivalent probes could be designed with respect to these regions, and the particular restriction enzyme and cDNA probe combination chosen below are arbitrary. However, it should be noted, as is understood in the art, that the efficacy of the probe is enhanced as it moves closer to the site of the polymorphism. Otherwise, additional, cleavage points may be encountered between the polymorphism and the probe, and also the probing site may be separated from the site of the polymorphism during replication by crossing-over events.

### F.1. Procedures for Analysis

Leukocytes were obtained from freshly drawn blood collected from each of the human subjects, and high molecular weight genomic DNA was isolated by the procedure of Law, D. J., et al, Gene (1984) 28:153-158.

High molecular weight DNA was divided into portions and each was digested to completion with one of the various restriction enzymes under conditions recommended by the suppliers (New England Biolabs and Bethesda Research Laboratories). The digests were electrophoresed in horizontal agarose gels in 30 mM  $\text{NaH}_2\text{PO}_4$ , 36 mM Tris, 1 mM EDTA, pH 7.7. After electrophoresis, DNA fragments were denatured in situ in 0.5 M NaOH/1.5 M NaCl for  $2 \times 10$  min, neutralized in 1 M ammonium acetate pH 7.2 for  $2 \times 10$  min, and transferred overnight onto nitrocellulose paper (Schleicher and Schuell). The filters were rinsed in  $2 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl, 0.015 M sodium citrate pH, 7.4) and baked for 2 hr at  $80^\circ\text{C}$  in vacuo and then were prehybridized for 5 hr in plastic bags using 0.3 ml/cm<sup>2</sup> of a solution containing  $5 \times$  SSPE ( $1 \times$  SSPE is 10 mM Na phosphate pH 7.4, 0.18 M NaCl and 1 mM EDTA) containing  $5 \times$  Denhardt's solution ( $1 \times$  Denhardt's contains 0.2 mg/ml each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 40% (vol/vol) formamide, and 250  $\mu\text{g/ml}$  sheared and denatured salmon sperm DNA, and hybridized overnight in the same bag in 0.1 ml/cm<sup>2</sup> of  $5 \times$  SSPE,  $1 \times$  Denhardt's solution, 40% (vol/vol) formamide, 10% dextran sulfate, and 100  $\mu\text{g/ml}$  sheared and denatured salmon sperm DNA, mixed with 100 ng per bag (containing 1 or 2 filters) of the appropriate  $^{32}\text{P}$ -labeled probe, as discussed below. Prehybridization and hybridization were performed at  $42^\circ\text{C}$ .

Filters were then washed twice at room temperature in  $2 \times$  SSC and twice at  $65^\circ\text{C}$  in  $2 \times$  SSC,  $1 \times$  Denhardt's solution. DNA sequences hybridized to the  $^{32}\text{P}$ -labeled probes were visualized by autoradiography using XAR-5 films (Kodak) and Cronex intensifying screens (Dupont) at  $-70^\circ\text{C}$  for 18 hr to 2 days.

Various specific probes are relevant to the illustrations below: a "first" apoA1 probe designated apoA1<sup>1</sup>, a "second" apoA1 probe designated apoA1<sup>2</sup>, p5'A1 probe, and apoCIII probe relevant to the apoA1/CIII/AIV complex; apoAII probe; 5'-apoB probe and 3'-apoB probe, relevant to apoB; apoC1; and pLDLR-3 probe.

The apoA1<sup>1</sup> probe is a 600 bp cDNA fragment which had been cleaved with *Avall* to remove terminal A/T tails, as described by Seilhamer, J.J., et al; DNA (1984) 3:309-317 (supra) incorporated herein by reference. The probe was labeled to a specific activity of  $2.0\text{-}5.0 \times 10^8$  cpm/ $\mu\text{g}$  by nick-translation, using the BRL nick-translation kit (Bethesda Research Laboratories) under recommended conditions with  $\alpha$  [ $^{32}\text{P}$ ] dGTP and  $\alpha$  [ $^{32}\text{P}$ ] dCTP (800 Ci/mmol; Amersham Corporation) in the presence of unlabeled dATP and dTTP. The probe was denatured just before the hybridization step by incubation for 5 min in a boiling water bath, followed by rapid cooling in ice water.

The apoA1<sup>2</sup> probe (also sometimes referred to herein as "HSAI probe") is a 1.0 kb *HindIII*/*SstI* cDNA subfragment prepared from a DNA fragment isolated from a human genomic library. The isolated fragment contained, in order, 6 kb of sequence 5' to the apoA1 gene, the entire apoA1 gene, 2.6 kb of intergenic region between the apoA1 and apoCIII gene, and 1.6 kb of apoCIII gene. This fragment was digested with *HindIII* and *SstI* and smaller fragments isolated. The 1.0 kb *HindIII*/*SstI* fragment designated apoA1<sup>2</sup> resulting from the digestion process corresponds to the 5' half of the apoA1 gene.

The p5'A1 probe was prepared as follows. A 12.5 kb *EcoRI*/*EcoRI* fragment was isolated from a human genomic library as described above. This fragment was digested with *HindIII* and *SstI* as above. A 1.4 kb *SstI*/*HindIII* fragment resulting from this digestion contained a unique sequence appropriate as a hybridization probe and was designated p5'A1. It was labeled as described above. The p5'A1 probe hybridizes close to the atherosclerosis deletion polymorphism described below, which is 4 kb 5' of the apoA1 gene. Restriction endonucleases other than *XmnI* could thus be used to detect this deletion, some giving fragments of smaller length which are more easily separated on agarose gel and easier to visualize.

The apoCIII probe is a 480 bp *SphI*/*pvuII* partial cDNA segment described by Levy-Wilson, B., et al, DNA (1984) 3:359-364.

The apoAII probe is a cDNA of 281 bp having the sequence shown as nucleotide #12 to nucleotide #290 in the sequence disclosed by Moore, et al, Biochem Biophys Res Comm (1984) 123:1-7, (supra), incorporated herein by reference.

The 5'-apoB probe, disclosed in copending application PCT/US86/02048, is a 0.97 kb *EcoRI*/*EcoRI* insert fragment which contains 70 bp of 5' untranslated region and 900 bp of sequence encoding the 30 kd protein. This probe, however, does not overlap either of the published apoB clones described by Deeb, S.S., or Lusis, A.J. (supra). Isolation of the *EcoRI* fragment used as probe is described by protter, A.A., et al, Proc Natl Acad Sci (USA) (in press), and the complete DNA sequence of the 5'-apoB probe is shown in Figure 2.

In addition, a 3.1 kb genomic DNA fragment designated 3'-apoB probe is described by Scott, et al,

Science (1985) 230: 37-43. It extends from an internal EcoRI site to almost the 3' end of the gene.

The apoCII probe is a 300 bp cDNA with the sequence shown spanning nucleotide #50 to nucleotide #352 in the sequence of apoCII cDNA, by Knott, et al, Nucleic Acids Res (1984) 12 :3909-3915 (supra), incorporated herein by reference.

5 The pDLR-3 probe is a portion of the full length (5.1 kb) human low density lipoprotein receptor-encoding DNA of Yamamoto et al, Cell (1984) 39: 27-38 (supra), incorporated herein by reference. The probe was prepared by cutting the full-length DNA fragment with XbaI and SmaI. This generates a 2.8 kb fragment from the 5' end and eliminates repetitive sequences located in the 3' untranslated region that would otherwise interfere with the hybridization (this probe was a gift from Yamamoto).

10

## F.2 Atherosclerosis-Associated Polymorphisms

15 The XmnI/8.2 kb polymorphism has been shown to be highly predictive of atherosclerosis, and has been shown to be a 300 bp deletion about 4 kb 5' of the apoAII gene. The polymorphism can be detected using XmnI digestion and probing with either apoAII probe or with the p5'AII probe; the presence of an 8.2 kb fragment shows the polymorphism is present. The same polymorphism can also be detected with other protocols, and the pattern which results depends on the restriction enzyme chosen and probe used to detect the digested fragments, as shown in the summary of Table 1. Table 1 shows the results obtained 20 when DNA obtained from individuals heterozygous for the XmnI/8.2 kb polymorphism were digested with series of additional enzymes and probed with p5'AII probe. The table shows the length of the fragment associated with the normal genotype, and the fragment length obtained for the polymorphism.

25

Table 1  
Additional Procedures to Detect  
the Atherosclerosis Deletion

30	<u>Probe</u> apoAII or	<u>Restriction</u> <u>Enzyme</u>	<u>More Common</u> <u>Allele (kb)</u>	<u>Less Common</u> <u>Allele (kb)</u>
	p5'AII	XmnI	8.5	8.2
	p5'AII	ApaI	3.5	3.2
	p5'AII	BglI	6.2	5.9
35	p5'AII	BstEII	2.5	2.1
	p5'AII	MspI	4.0	5.9
	p5'AII	PvuII	1.9	1.6
	p5'AII	RsaI	3.5	3.2
40	p5'AII	StuI	5.8	5.5
	p5'AII	TaqI	5.2	9.1
	p5'AII	BglIII	3.2	2.9
	p5'AII	HindIII	2.7	2.4
	p5'AII	KpnI	4.9	4.6
	p5'AII	MstII	5.7	5.4
45	p5'AII	NcoI	4.5	4.2
	p5'AII	SspI	2.8	2.5

50 The presence of the polymorphism which results in the generation of an 8.2 kb fragment upon XmnI digestion (or the results indicated on Table 1) correlates significantly with atherosclerosis. As described above, all individuals exhibiting this polymorphism in their genomes are not only affected by atherosclerosis but also exhibit severe clinical symptoms. However, the converse is not true: atherosclerosis may occur in individuals who lack this genetic abnormality. None of the individuals tested were homozygous with respect to this genetic feature; all contained an allele which generated the "normal" 8.5 kb fragment.

55 Additional polymorphisms can also be found which correlate in some way with the risk of atherosclerosis. To determine these polymorphisms, control and patient groups are set up using as a criterion positive or negative results relating to atheromatous plaque formation as determined by angiography. Persons are classified as "patients" who show plaques in this assay, whether or not they have had suffered heart

attacks. They are designated "controls" if the results of this test are negative; none of these persons will have had a heart attack.

In interpreting the results, a standard x-square analysis is used to determine a significance level. The significance level represents the probability that the results obtained would not hold up if high numbers of subjects were used. For example, a significance level of less than 0.05 means that there is a greater than 95% probability that testing additional or larger numbers of subjects would yield the same results; a significance level of 0.10 means that there is one chance in 10 that the results would be different if a larger or different sample were tested.

The findings are interpreted in terms of the relative risk of persons having the polymorphism to show the disease, compared to those having an absence of the polymorphism. These "relative incidence" values are calculated according to Wolf, B., Ann Hum Genet (1955) 19:251. As applied to the assays below, the relative incidence is calculated as equal to:

$$\frac{PP \times CN}{PN \times CP}$$

where

- PP is the number of patients having the polymorphism;
- PN is the number of patients not having the polymorphism;
- CP is the number of controls having the polymorphism;
- CN is the number of controls not having the polymorphism.

The value calculated by this ratio, if significantly greater than 1, indicates that the persons having the polymorphism are at a greater risk of having the disease; a value less than 1 shows protection against the disease.

In addition to the deletion associated with atherosclerosis and heart attack outlined above, the polymorphisms of the invention are also present in the apoAI-CIII-AIV gene complex or apoAII, apoB, apoCII or LDL-R genes. The general pattern of the polymorphisms in the apoAI-CIII-AIV complex region is shown in Figure 1.

Table 2 shows numerous polymorphisms, including those of the invention, associated with apoAI/CIII/AIV; apoAII, apoB, apoCII, and LDL-R.

Table 2

	Polymorphism	Location of Polymorphism	Probe	Enzyme	More Common Allele, kb	Less Common Allele, kb	Freq.**
5	XmnI/8.2 kb	4 kb 5' of apoAI gene	p5'AI apoAI <sup>1</sup>	XmnI	8.5	8.2	0.03 ± 0.01
	BanI-apoAI	3rd intron of apoAI gene	apoAI <sup>1</sup>	BanI	0.82 0.64	1.46	0.16 ± 0.02
10	MspI/0.73-apoAI	1st intron of apoAI gene, 40 bp inside	apoAI <sup>1</sup>	MspI	0.65	0.73	0.25 ± 0.02
	BanII-apoAI	3rd intron of apoAI gene	apoAI <sup>2</sup>	BanII	0.274	0.452	0.09 ± 0.02
15	BsmI-apoAI	3rd intron of apoAI gene	apoCIII apoAI <sup>1</sup>	BsmI	-- 1.3, 5.6	9.3 6.9	0.05 ± 0.01 0.05 ± 0.01
	BstXI-apoAI	1.0 kb 3' of apoAI gene	apoCIII, apoAI <sup>1</sup>	BstXI	1.8 2.7	4.5 4.5	0.15 ± 0.02 0.15 ± 0.02
	BstEII-apoAI	--	apoAI	BstEII	6.5	4.8	0.05 ± 0.04
20	HaeIII-apoB	--	3'-apoB	HaeIII	--	0.9-1.1	--
	MvaI-apoB	3' end of apoB gene	3'-apoB	MvaI	0.76	0.96	0.04 ± 0.01
	NsiI-apoB	--	5'-apoB	NsiI	6.2	3.8	0.10 ± 0.01
25	HpaI-apoB	--	5'-apoB	HpaI	--	15.1	0.05 ± 0.01
	BglI-apoCI	--	apoCI	BglI	6.2	8.7	0.03 ± 0.01
	DraI-apoCI	--	apoCI	DraI	7.6, 2.6	10.2	0.22 ± 0.02
	TaqI-apoCI	--	apoCI	TaqI	--	5.9	0.05 ± 0.01
30	CfrBI-LDL-R	--	pLDLR-3	Cfr13I	4.0	3.0	0.10 ± 0.02
	BstEII-LDL-R	--	pLDLR-3	BstEII	33	22.11	0.25 ± 0.05

<sup>1</sup> first apoAI probe

<sup>2</sup> second apoAI probe, or "HSAI" probe

\*\* error calculated as standard error of the mean

35 The polymorphisms of the invention tabulated above are as follows:

The BanI-apoAI polymorphism of the apoI gene is located in the third intron of the apoAI gene, about 260 bp downstream of the "MspI" polymorphism shown on the map of Figure 1. The majority (84%) of genomic DNA alleles digested with BanI and probed with apoAI probe, show a pair of fragments of 0.82 kb and 0.64 kb. A small proportion (16%) give a single 1.46 kb fragment.

40 The MspI/0.73-apoAI polymorphism of the apoAI gene is located in the first intron of the apoI gene (previously believed to be located in the 3'untranslated region; see U.S. Serial No. 924,911), about 40 bp inside of the beginning of the gene as shown on the map of Figure 1. The majority (75%) of genomic DNA alleles digested with MspI and probed with apoAI probe, show a fragment of 0.65 kb. A small proportion (25%) give a 0.73 kb fragment.

45 The BanII-apoAI polymorphism of the apoAI gene is located in the third intron of the apoAI gene, about 140 bp downstream from the BanI-apoAI polymorphism. The majority (91%) of genomic DNA alleles digested with BanII and probed with the HSAI (ApoAI<sup>2</sup>) probe described above show a fragment of 0.274 kb. A small proportion (9%) give a 0.452 kb fragment.

50 The BsmI-apoAI polymorphism of the apoAI gene is located in the third intron of the apoAI gene about 200 bp 3' of the Msp-apoAI polymorphism. It is detected against a constant background of a 18.0 kb fragment. The majority (95%) of genomic DNA alleles digested with BsmI and probed with apoAI<sup>1</sup> probe showed a fragment of 5.6 kb, while a small proportion (5%) gave a 6.9 kb fragment. When apoCIII is used as probe, 5% of the alleles yield a 9.3 kb fragment absent in the remaining 95%.

55 The BstXI-apoAI polymorphism of the apoAI gene is located about 1.0 kb 3' of the apoAI gene and detected against a constant background of 0.8 kb and 1.2 kb. The majority (85%) of genomic DNA alleles digested with BstXI and probed with apoCIII probe showed a 1.8 kb fragment, while a smaller proportion (15%) gave a 4.5 kb fragment. When ApoAI<sup>1</sup> is used as probe, the 95% of alleles give a 2.7 kb fragment, the remaining 15% a 4.5 kb fragment.